

# pH dependence of the formation of an M-type intermediate in the photocycle of 13-*cis*-bacteriorhodopsin

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An M-type intermediate is formed in the 13-*cis*-bR photocycle in purple membranes at high pH. This is presumably due to deprotonation of the same group whose deprotonation causes a large increase in rate of M formation in the *trans*-bR photocycle (the 'alkaline transition'). For Triton X-100-solubilized bR, the alkaline transition is shifted to a lower pH value by more than 2 pH units. The alkaline transition in Triton-solubilized preparations changes the efficiency of the M intermediate formation in the 13-*cis*-sbR photocycle. The M intermediate formation in 13-*cis*-sbR, as in the case of *trans*-sbR, is completely inhibited when the blue 'acidic' bR is formed at low pH. The protonation state of the group affecting formation of the M intermediate in 13-*cis*-bR at high pH and the group which is responsible for the transition to the blue acidic form influence in a similar way the equilibrium between bR isomers in the dark-adapted form as well as the rate of dark adaptation.

Bacteriorhodopsin; Photocycle; M intermediate; pH dependence; Light-dark adaptation

## 1. INTRODUCTION

Bacteriorhodopsin (absorption maximum of initial light-adapted form ~570 nm) is a light-driven proton pump in the purple membrane of halobacteria. The photocycle of *trans*-bR is accompanied by formation of a short wavelength (~410 nm) absorbing deprotonated intermediate 'M' and by transmembrane proton transfer [1,2]. We have shown that illumination of 13-*cis*-bR in pm at high pH also results in formation of an M-type intermediate and proton transfer [3,4]. The pK of this transition decreases when 13-*cis*-bR is incorporated into liposomes or solubilized with Triton X-100. The question arises about what processes underlie the pH-dependent transition. On the one hand, the pH dependence may be due to the pK of the Schiff base in the excited 13-*cis*-bR molecule (this idea was proposed for the 13-*cis*-bR analogs in [5]). On the other hand, it may depend on the pK of some protolytic group of the protein, which can serve as an acceptor of H from the Schiff base or as a regulator of this process.

In the present paper we conclude that the pH dependence of M formation in the 13-*cis*-bR photocycle is due to the protonation/deprotonation state of a moiety of the protein which also influences the kinetics of the M intermediate formation during the *trans*-bR photocycle as well as the equilibrium between two isomeric forms in dark-adapted pm and the rate of dark adaptation.

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**Abbreviations:** 13-*cis*-bR, *trans*-bR, bacteriorhodopsins containing 13-*cis* and all-*trans* retinals; d-sbR and l-sbR, dark-adapted and light-adapted bR solubilized with 2% Triton X-100; pm, purple membrane.

## 2. MATERIALS AND METHODS

In all the experiments pm preparations from *Halobacterium halobium* ET 1001 showing retarded transition at high pH to the form with a differential maximum at 460 nm were used. The laser-flash-induced spectral responses of pm and sbR were measured as described previously [3,4].

The light intensity of laser flashes (532 nm, 15 ns) was 15 mJ/cm. The bR concentration was 12 mM in a 1-cm cuvette. The absorption spectra and the rate of dark adaptation were measured with a Hitachi U-3400 spectrophotometer. All experiments were performed at 20°C.

For the dark adaptation, pm at pH 6.5 and sbR at pH 7.5 were kept for 24 h in the dark at room temperature. The pH dependencies shown in Fig. 1A and 2A were determined by measuring the kinetic response to a single light flash immediately (not longer than 30 s) after rapid adjustment of the pH change to the indicated value. The light adaptation was performed at the same pH as the dark adaptation, and then the pH was changed to the indicated value. The peculiarities of sbR light adaptation were described earlier [4].

## 3. RESULTS AND DISCUSSION

In pm, as the pH is increased 13-*cis*-bR gains the capability to form an M-type intermediate with a pK of 8.45 at high ionic strength (Fig. 1A). If some protein group is responsible for this process, it is reasonable that deprotonation of this group would influence the *trans*-bR photocycle. Indeed, it is well known that at high pH values the efficiency of M intermediate formation in the *trans*-bR photocycle does not change, but the rate of M formation increases [7,8] (the half-rise time decreases by more than 50 times). However, the pK of this transition is 0.2–0.3 pH units higher than the pK of the pH dependence of the formation of the M intermediate in the 13-*cis*-bR photocycle (Fig. 1A). Both pH dependencies are synchronously shifted to a higher pH with decreasing ionic strength. It is possible that the

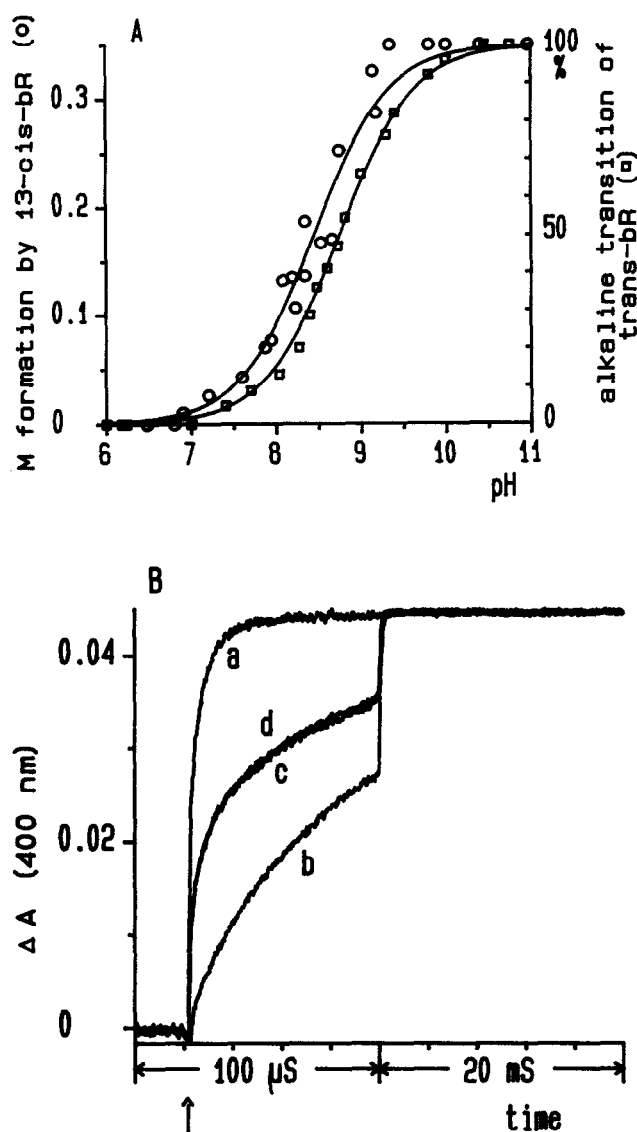


Fig. 1. (A) pH-dependence of the efficiency of the M intermediate formation by 13-*cis*-bR and *trans*-bR transition from neutral to alkaline form in pm. For the left ordinate axis the amplitude of the optical response at 400 nm of the equivalent quantity of *trans*-bR at pH 6.0 was taken to be unity. The M intermediate formation by 13-*cis*-bR at the indicated pH was determined as the difference between the amplitude of the response of dark-adapted pm and the amplitude of the response of the *trans*-bR form involved in this response. Solid curves represent theoretical titration curves for groups with pK 8.45 and 8.75, respectively. (B) The illustration of the method for determining the extent of the *trans*-bR transition from the neutral to the alkaline form. The decay components were subtracted from all the photoresponses. a, pH 9.8; b, pH 6.5; c, pH 8.7; d, photoresponse, obtained as the sum of the a and b responses ( $d = 0.47 \times a + 0.53 \times b$ ). The sample contained 0.5 M NaCl, 5 mM MES, HEPES, TRIS, CHES, CAPS. Here and in Fig. 2 the vertical arrow indicates the moment of a laser flash.

difference in the pK values is due to the differing environment of this group in 13-*cis*-bR and *trans*-bR (for example, to different distances from the protonated Schiff base). The identity of these groups is confirmed by data obtained for sbR.

Under neutral pH conditions, the parameters of the sbR photocycle are much like those of the pm photocycle at high pH values. The pH-dependent transitions of the 13-*cis*-bR and *trans*-bR photocycles similar to the above ones were found for sbR but at rather low pH values (Fig. 2A). The efficiency of M intermediate formation during the 13-*cis*-bR photocycle changes at pK 6.2–6.4. Simultaneously, the rate of the M intermediate formation for the *trans*-bR photocycle changes over the same pH range (Fig. 2B). However, acceleration of the M intermediate formation in the *trans*-bR photocycle is less marked than in the case of pm (the half-rise time changes only by 5 times). The efficiency of the M intermediate formation does not change as in the case of pm. The essential distinction of sbR from pm concerns the efficiency of the M intermediate formation by 13-*cis*-bR. At neutral pH values in pm, 13-*cis*-bR does not form the M intermediate and the efficiency of its formation after the alkaline transition reaches only 33–36% of that of the M intermediate formation by *trans*-bR at pH 6. In contrast, 13-*cis*-sbR and *trans*-sbR give M intermediates with an equal efficiency at pH above 7. The analog of alkaline transition only alters the efficiency of M intermediate formation in the 13-*cis*-sbR photocycle from 0.7 to 1 from the efficiency of M formation by *trans*-bR. The ability of 13-*cis*-sbR to form the M intermediate at pH < 6 follows from (i) the comparison between the amplitudes of photoresponses with isomer content in D-sbR and L-sbR, and (ii) the clear cut distinction between the kinetics of D-sbR and L-sbR photoresponses at 400 nm (not shown). 13-*cis*-sbR and *trans*-sbR lose the ability to form the M intermediate synchronously with the transition of sbR to the blue acidic form at low pH values. This phenomenon is well known for *trans*-bR from pm [1,2].

It is notable that the alkaline transition affects the photochemical conversions of 13-*cis*-sbR and *trans*-sbR to a smaller degree than in the case of pm and, vice versa, its influence on the absorption spectrum is more pronounced in the case of sbR. We saw that under the alkaline transition the *trans*-bR absorption maximum in pm is 1–2 nm shifted to the long wavelength region. These data are in good agreement with the previous results [7]. Simultaneously, it is seen that the 13-*cis*-bR absorption maximum is 1 nm shifted to the shortwave and its extinction slightly decreased. At the same time, the absorption maximums of 13-*cis*-bR and *trans*-bR are 3–4 nm shifted to the short wavelength region with a slight decline in the extinction.

The two above (acidic and alkaline) transitions, affecting the M intermediate formation in the *trans*-bR and 13-*cis*-bR photocycles are revealed in some processes proceeding in the dark. We have shown earlier [8] that the *trans*-bR/13-*cis*-bR ratio in dark-adapted pm varies depending on pH values, at which dark adaptation takes place. The 13-*cis*-bR content decreases from 67–68% to 55% with the lowering pH in the same pH

region where the protein group, responsible for the M intermediate formation in the 13-*cis*-bR photocycle, is protonated. The 13-*cis*-bR content decreases to 35% again whereas protonation of the group responsible for the M intermediate formation in the *trans*-bR photocycle and for the protein transition to the blue acidic form is enhanced. In sbR a sufficient variation in the *trans*-bR/13-*cis*-bR ratio is observed only during transition to the blue acidic form (the *trans*-bR content increases as in the case of pm). The results presented in [8] were obtained from analysis of the bR photocycle. However, now we have confirmed these data using the method described in [9]. Moreover, simple experiments provide qualitative evidence for the change in the *trans*-bR/13-*cis*-bR ratio: pm dark-adapted at pH 10 and 2, after fast pH return to 6, has a 4–5 nm red-shifted absorption maximum (pH 10 → pH 6), and a 4–5 nm blue-shifted

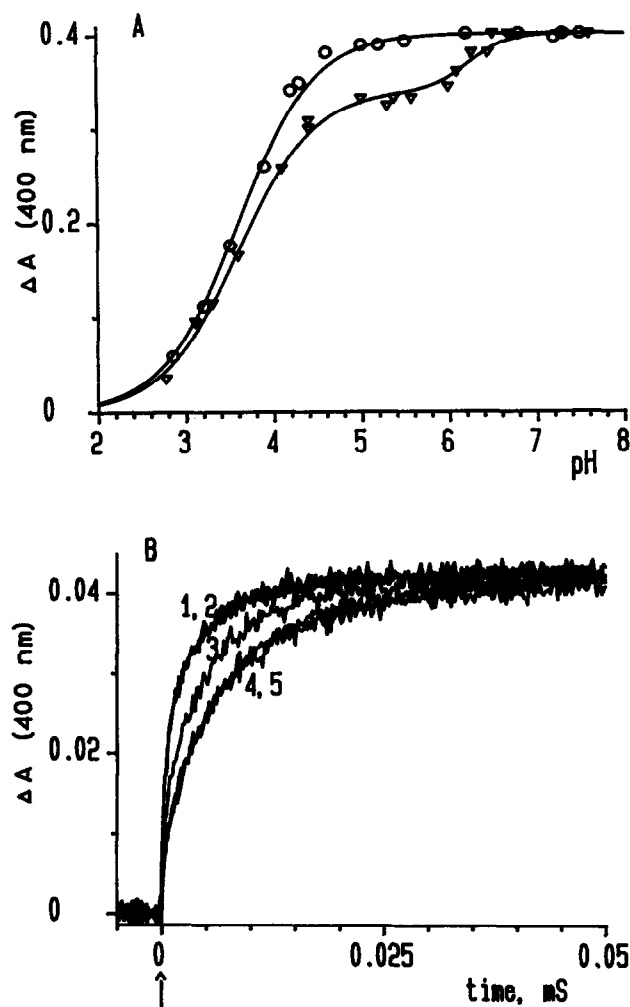


Fig. 2. (A) pH-dependence of the M intermediate formation in dark-adapted (▽) and light-adapted (○) sbR preparations. (B) pH-dependence of kinetics of the M formation in light-adapted sbR. 1, pH 7.3; 2, pH 7.9; 3, pH 6.45; 4, pH 5.3; 5, pH 4.3. The sample contained 2% Triton X-100, 100 mM NaCl, 5 mM K-citrate-phosphate-borate buffer.

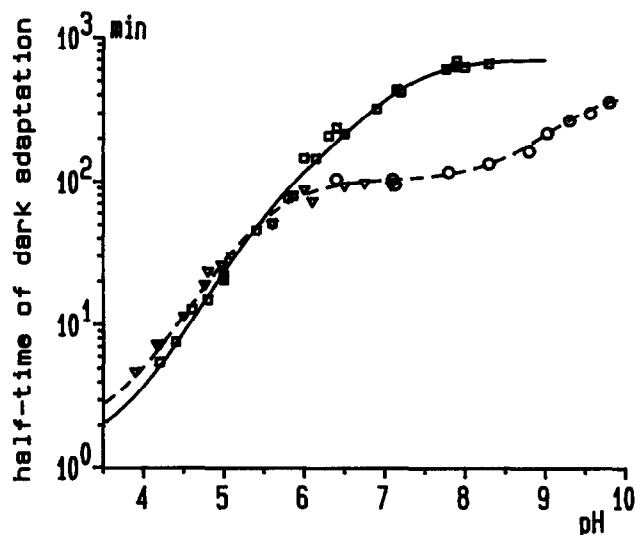


Fig. 3. pH-dependence of the dark-adaptation rate in pm (▽, ○) and sbR (□). Solid and dashed curves represent theoretical curves, constructed according to the model developed in [11], with the assumption that only two ionizable groups participate in catalysis. For pm  $pK = 3.7$ ,  $pK = 8.6$ , the rate constants for different dark-adapted forms are equal to 0.0068, 0.00011 and 0.00026  $s^{-1}$ , respectively. For sbR  $pK = 3.7$ ,  $pK = 6.4$ , the rate constants are equal to 0.0094, 0.00009, 0.000017  $s^{-1}$ . The sample contained: ▽, 2 mM K-citrate-phosphate-MES buffer; ○, 0.5 M NaCl, 5 mM MES, HEPES, TRIS, CHES, CAPS; □, 100 mM NaCl, 5 mM K-citrate, phosphate, MES, HEPES, TRIS.

one (pH 2 → pH 6) as compared with the absorption maximum of pm dark-adapted at pH 6.

The protonation of the two groups also influences the rate of dark equilibration. This feature of the dark adaptation for pm was described earlier [10,11] and is confirmed by our experiments as well (Fig. 3). The  $pK$  values of the groups lowering the activation energy of isomerization fit well with the  $pK$  of the groups responsible for the acidic and alkaline transitions. It should be noted that the pH-dependence of the kinetics of adaptation in sbR (described by a theoretical curve, suggesting the participation of two groups with  $pK$  3.7, and 6.4 (Fig. 3) in catalysis) agrees well with the assumption about the shift of sbR alkaline transition to the low pH region. These groups can readily be imagined to participate in adaptation catalysis if we suggest that they are localized not far from the chromophore group of the Schiff base, and that protonation of one group leads to the growth of the Schiff base charge and to the increase in the coupling of the polyene chain near the N atom. Consequently, the order of the bonds between C13 and C14, and between C15 and N is decreased and isomerization about these bonds is facilitated by analogy with the model Schiff bases [12].

Hence, the results suggest the existence of two protein groups, the protonation of which determines the Schiff base deprotonation during the 13-*cis*-bR photocycle. Protonation of any of these groups facilitates isomeriza-

tion about the double C13=C14 and C=N bonds and stabilizes *trans*-bR in comparison with 13-*cis*-bR. Deprotonation of the group with low pK (probably Asp<sup>85</sup> [13]) leads to the appearance of the M form in the *trans*-bR photocycle in pm, and in the 13-*cis*-sbR photocycle as well, but not in the 13-*cis*-bR photocycle in pm. This difference may be due to the different rigidity of the protein structure in various environments. The group which determines the alkaline transition possibly functions as an additional proton acceptor and accelerates deprotonation of the Schiff base in the *trans*-bR photocycle. It permits deprotonation of the Schiff base during the 13-*cis*-bR photocycle in pm or increases the efficiency of the M intermediate formation in 13-*cis*-sbR. According to the suggestion [6,7], one of the Tyr residues is a candidate for this role. The analogy of alkaline transitions in pm and sbR suggests the participation of one and the same group in these transitions. However, the rather low pK of the transitions in the sbR photocycle is in contradiction with Tyr participation in this process. A more appropriate candidate for this role could be a residue of the carboxyl-containing amino acid with enhanced pK, by analogy with the enhanced pK of Asp<sup>96</sup>.

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